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An Experimental Cholera in Adult Rabbits

—A new model for the analysis of diarrheal stool—

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Abstract: A new model for experimental cholera in adult rabbits was established with the following procedure. A soft elastic silicone tube was inserted to the terminal ileum through an incision at the ileo-caecal junction. The incision and the terminal ileum were tied with silk to fix the tube and to block the movement of the intestinal contents between the ileum and the cecum. A proper amount of *Vibrio cholerae* O1 was inoculated to the duodenum. Then, closed the abdomen, and the tube out side the body was fixed to the skin with sutures. After the inoculation, the diarrhea started in about 12 hours. The amount of the diarrheal fluid which was sucked through the tube was roughly about 20–30 ml per hour, under the hydrated condition with continuous intravenous fluid therapy. In this model, diarrheal fluid sucked through the tube is not modified in the cecum and the colon. Therefore, the accurate informations about the phenomena took place in the small intestine are obtained by the analysis of sucked stool (cholera stool in situ). We have applied this model for many experiments, and all animals without exception developed copious amount of diarrheal fluid. We designated this procedure the T-model.

Key Words: Experimental cholera, adult rabbit, T-model.

INTRODUCTION

The development of the disease model in animals make a great advance in our understanding of human diseases. A number of models for experimental cholera has been established, and they contributed to the investigation of cholera. In the ideal animal model, the animal would have a minimum of artificial manipulation, it woud develop the symptomes similar to the human disease with the similar causative agents, the course of the disease (from the onset to the converlescence) could be followed, sampling from the animals would always be possible with a simple technique, and the animals could readily be obtained and easily be handled. But an animal is animal and not a human being, concequently, it is almost impossible to make a really ideal model. Each of many models for experimental cholera established so far has played an important but a limited role to clarify the entity of cholera. For the studies on the mechanism of ac-

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tion of cholera toxin, on the efficacy of antibiotics and immunity, and on the adherence of *Vibrio cholerae* O1 to the intestinal mucosa, the model of infant rabbits and intestinal loop of adult rabbits have greatly contributed (De and Chatterjee, 1953; Dutta and Habbu, 1955; Finkelstein *et al.*, 1964; Kasai and Burrows, 1966; Finkelstein *et al.*, 1969; Freter, 1969; Nelson *et al.*, 1976; Srivastava *et al.*, 1980). Suckling mice cholera model has contributed to clarify the importance of passive immunization with milk of immune mother (Uji-iye and Kobari, 1970). But these models are far from the ideal. Although a large inoculum size (10^{11}) is necessary, canine model of cholera is closer to the ideal to some extent (Sack and Carpenter, 1969a, 1969b, 1969c). However, it is not easy to use the dogs because of labor involved. Chinchillas (Blachman *et al.*, 1974) may be too small to give intravenous fluid therapy to keep the animals alive, and are not readily available outside the USA and Canada. RITARD (Removable Intestinal Tie-Adult Rabbit Diarrhea) model may be more ideal (Spira *et al.*, 1981). But for the study on the analysis of diarrheal stool, the RITARD model is also not so ideal. Because the cholera stool originated from the small intestine must be modified in the unligated portion of the cecum and in the colon. The rabbit cecum may have a different function from the human cecum. And also the stool samples can not regularly be collected. In order to perform the analysis of cholera stool in situ with the serial sampling, a new model in the adult rabbits was figured out.

MATERIALS AND METHODS

Rabbits: Strain Japan White of either sexes donated by local breeders were used. All rabbits were apparently healthy, weighed 2−3 kg. The rabbits were starved but water ad lib for 24 hours prior to challenge.

Pathogen: *Vibrio cholerae* O1, biotype eltor, serotype Ogawa (our laboratory No. A−66), isolated from human stool in Kenya in 1980 was used throughout this study. The strain has been maintained in the butt of nutrient agar (Eiken) at room temperature with subcultures every 3 to 4 months since the isolation.

Preparation of the inoculum: From the stock tube, the strain was cultured on the nutrient agar plate (Eiken) at 37 C overnight. A colony was inoculated to the heart infusion broth (Eiken) in the test tube and incubated at 37 C for 4 to 5 hours. The culture was then diluted 100 folds with normal saline solution. One milli-litter of this dilution was used as the inoculum.

Model procedure: The model procedure is schematically shown in Figure 1. The rabbits were anesthesized with 1.5 ml of ketamine hydrocloride (intramuscular injection) and the continuous ether inhalation. The laparotomy was done under these anesthesia with local xylocain. At the ileo-caecal junction, a small incision was made and a soft elastic silicone tube (Fig. 2) was inserted through the incision, going up to the ileum.

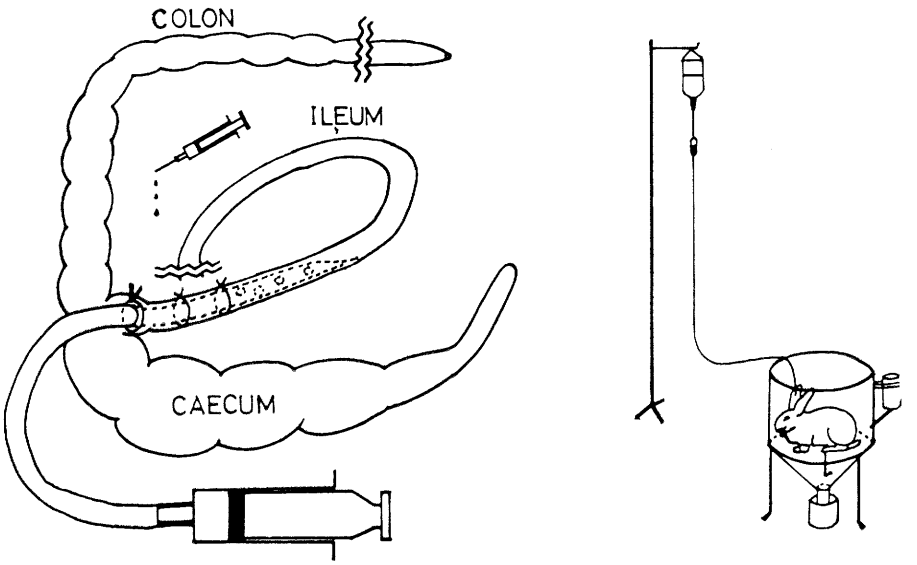

Fig.1. The schematical picture of model procedure.

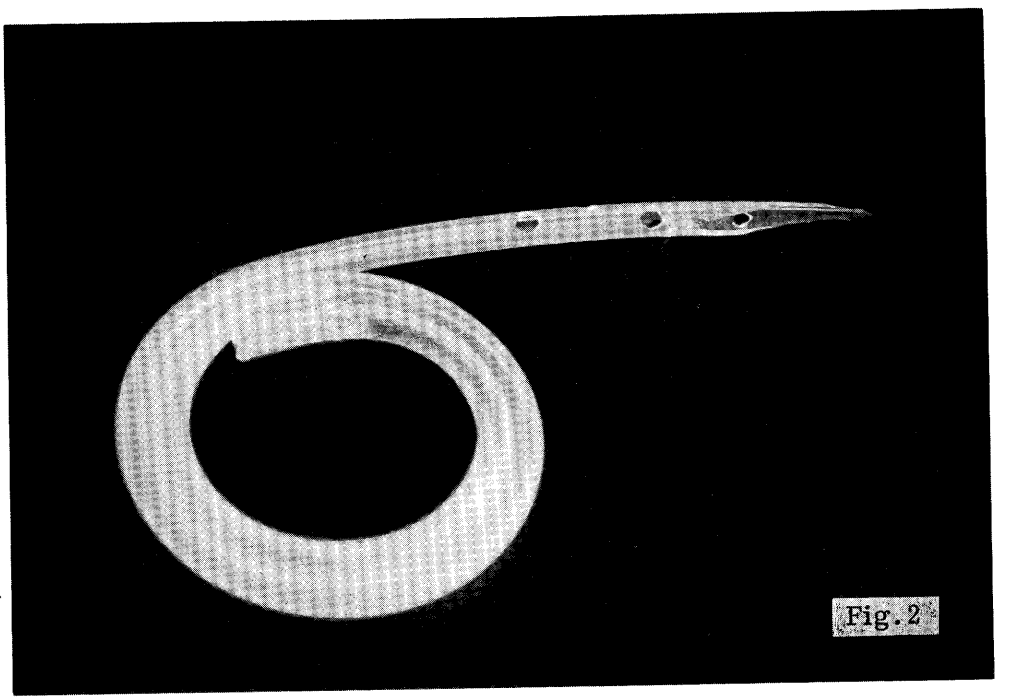

Fig.2. A tube applied in this model. The diameter of the cross section is 6 mm (3.5 mm inside). The tip is cut oblique to make the tube softer and to avoid obstruction. Side holes are made at the top area.

The tip of the tube was cut oblique to make the tube softer and to avoid the obstruction with the intestinal contents. The tip of the tube was located at about 10 cm from ileo-caecal junction. Then the incision was tied with silk surrounding the tube. The terminal ileum with the tube was also ligated to fix the tube and to block the movement of the intestinal contents between the ileum and the cecum. The actual procedure of the surgery was shown in the photographs (Fig. 3, 4, 5, 6). Inoculum was given intraduodenally. After closing the abdomen, the tube outside the body was fixed to the skin with sutures, and clipped the orifice of the tube. The trial suction was started in 6 hours after challenge.

Sampling method: An injectionsyringe was connected to the tube and sucked the diarrheal fluid. Usually the suction was repeated every hour. Every time, all amount of the fluid which could be sucked was taken out. The volume, color and character of the sucked diarrheal fluid were recorded. At the time of sacrifice, all of the small intestine was removed with the contents, and the retention fluid in the intestine was measured with the length of the whole intestine. Other than the stool sample, venous blood before challenge and the heart blood at the time of sacrifice were taken to measure the hematocrit values.

Analysis of the stool samples: Hydrogen ion concentrations were measured with the pH meter type COM-10 supplied by Denki Kagaku Keiki Co. Viable vibrio count was done on the TCBS agar plate (Eiken) with 10-fold dilution method. Cholera toxin concentration was titlated with reversed passive latex agglutination method. Before titlation the stool was centrifuged and filtered with 0.45 μ milipore membrane. Some of the titlation samples were randomly taken to the neutralization test with anti cholera toxin.

Fluid replacement for rehydration: For the first four cases (No. 1 to No. 4), intravenous shot with 5% glucose in lactated Ringer's solution was given at times depending on the signs of the rabbits. For the other eight cases (No. 5 to No. 12), intravenous route was taken with Gage No. 23 Venula, and the above solution was continuously given with drip infusion method. In general, the fluid therapy was started in 12 hours after challenge. About 100 ml of the fluid was given within the first hour as the initial hydration. And then 20 to 40 ml per hour was given depending on the amount of the sucked stool.

RESULTS

The rabbits undergone surgery and inoculation of the pathogen restored the healthy appearance within a few hours in their cage. In about 12 hours after challenge, the collaps of the blood vessels in the ears was observed. At that time, the diarrheal stool could usually be sucked through the tube.

The intervals between the challenge and the first suction of diarrheal stool ranged

Table 1. Amount of extracted stool and (i.v. fluid)

Case No.	Body weight rabbits (kg)	Intervals between inoculation and the first fluid suction	Time process			
			5-6	10-11	15-16	20-21 hours
1	3.0	15 hours	116 (70)	154 (145)	174 (145)	180 (145) ml
2	2.9	19	76 (205)	111 (305)	x	x
3	2.7	13	193 (160)	330 (380)	x	x
4	2.1	11	151 (155)	233 (265)	x	x
5	2.5	11	70 (160)	177 (350)	307 (490)	427 (590)
6	2.3	11	345 (370)	618 (740)	804 (950)	928 (1,130)
7	2.2	12	161 (350)	224 (470)	354 (550)	499 (790)
8	2.1	10	213 (280)	356 (450)	452 (600)	595 (700)
9	3.1	12	282 (250)	551 (600)	685 (780)	873 (930)
10	2.8	10	120 (210)	209 (320)	264 (500)	x
11	2.5	11	98 (260)	129 (380)	150 (450)	186 (650)
12	2.7	8	122 (150)	246 (400)	352 (680)	456 (900)
Average	2.58	11.9	162 (218)	278 (400)	393 (572)	518 (729)

Cases No.1 to No.4 were treated with repeated i.v. shot fluid.
Cases No.5 to No.12 were treated with continuous i.v. drip infusion fluid.
x: Animals died.

Table 2. Analysis of the extracted stool

Case No.	Initial stool			Stool at about 20th hour		
	pH	viable vibrio count	cholera toxin	pH	viable vibrio count	cholera toxin
1		ND			ND	
2	8.22	8×10^8/ml	1,000 ng/ml	8.04	2×10^9/ml	1,000 ng/ml
3	8.00	2×10^9	1,000	7.25@	3×10^8 @	4,000@
4	7.53	2×10^9	500	7.28@	3×10^9 @	2,000@
5	7.91	1×10^9	1,000	6.96	2×10^9	250
6	8.18	6×10^8	1,000	8.02	4×10^8	62.5
7	7.85	7×10^8	62.5	7.87	4×10^8	7.8
8	8.14	2×10^8	2,000	7.59	9×10^7	31.3
9	8.58	1×10^8	ND	8.30	3×10^8	ND
10	ND	ND	ND	ND	ND	ND
11	8.63	8×10^8	8,000	8.01	9×10^7	125
12	8.15	7×10^8	500	7.38	2×10^8	4,000

@: Stools at about 10th hour

from 8 to 19 hours. The amount of the sucked stool was roughly about 20 to 30 ml per hour in average (Tab. 1). But in the case No. 6, 928 ml of watery stool came out within 20 hours (46.4 ml per hour). In the beginning, the sucked stool contained much amount of food sediment. But as passing the time, the stool was changing to clear watery (Fig. 7).

The number of viable vibrio in the sucked stool ranged constantly from 10^8 to 10^9/ml. Cholera toxin concentration in the stool was variable, but most frequently, it was about 1,000 ng/ml. Although this vibrio (A-66) produced only 31.3 ng/ml of cholera toxin *in vitro* (syncase medium, 30 C, 24 hours), the maximum production *in*

Table 3. The amount of retained fluid in the intestine at the time of sacrifice

Case No.	Amount of fluid, ml
1	120
2	200
3	100
4	100
5	100
6	70
7	175
8	150
9	100
10	80
11	ND
12	140

Table 4. Hematocrit values of the animals

Case No.	Venous blood before inocul.	Heart blood at sacrifice
1	ND	ND
2	ND	ND
3	41	54
4	40	54
5	43	52
6	41	33
7	47	38
8	45	50
9	42	43
10	41	39
11	39	ND
12	40	30

vivo was 8,000 ng/ml (case No. 11) as shown in Table 2.

The fluid in the intestine could not be sucked completely out. When the animals were sacrificed, a large amount of fluid was still seen in the intestine. The volume of the remaining fluid in the small intestine was 121 ml in average (Tab. 3). The length of the small intestines was more or less 250 cm.

The hematocrit values before challenge and at the time of sacrifice are shown in Table 4. In the cases No. 3 and No. 4, the volume of i. v. fluid minus the volume of sucked stool were 50 ml and 32 ml respectively. And the hematocrit values elevated from 41% to 54%, and from 40% to 54% respectively. While in the case No. 10, the volume of i. v. fluid was 246 ml over the volume of sucked stool. And the hematocrit value changed from 41% to 39%. In the cases with enough hydration, the urination was seen at times. From these hematocrit values and the amount of remaining fluid in the intestine, the volume necessary for the initial hydration was estimated as about 100-200 ml. The hematocrit values in the rabbits with intestinal loop model ranged from 54% to 61% when the animals were sacrificed in 11 hours after challenge.

DISCUSSION

In this new model (the T-model), a large amount of stool specimen is hourly obtained without quality modification in the cecum and the colon. It is quite beneficial for the study on the original cholera stool from the small intestine. It is possible to study not only on the composition of the diarrheal stool in situ such as enzyme or other proteins including antibodies, electrolytes, bile salts, cell materials, etc., but also on the absorption and secretion of artificially given materials. And, if required, it is possible to make animals survive for a long time by removing the tube. The RITARD

model (Spira *et al.*, 1981) was established for experimental diarrhea due to *Vibrio cholerae* O1 and enterotoxigenic *Escherichia coli*. Although we had not yet examined the *E. coli* in this new model, it is almost sure that the *E. coli* also develop diarrhea because of similar model procedure.

The i. v. fluid is necessary to continue sampling. Without fluid therapy, the animals will die very soon after the diarrhea through the tube is seen. The amount of i. v. fluid depends on the volume of sucked stool. But a large amount of fluid is remained in the intestine and the stomach even just after sucking. Therefore about 100–200 ml of the fluid should be added to the volume of sucked stool.

The pH of the stool ranged from 7.25 to 8.63, consequently, the i. v. fluid should be alkaline. Actually lactated Ringer's solution was used in this study, and succeeded. The glucose is necessary because of starvation. If glucose is not given, the animals become so weakened during the experiment lasting for a few days. This therapeutic method is quite similar to that for human cholera.

In human cholera, the fluid amount for initial hydration is calculated by the following formula (Barua and Burrows, 1974);

(Plasma specific gravity -1.025) $\times 4 \times$ body weight (kg) litter.

In this model, it is quite difficult to take plasma from the dehydrated animals. But hematocrit value could be measured. So, if necessary, the following formula could be substituted for this model;

(hematocrit value -40) $\times 5 \times$ body weight (kg) mili-litter.

Because of keeping i. v. route, we can control the water and electrolytes balance of the animals. Therefore, this model can also be used for the study on the physiological reactions under dehydration and its convalescence to normally hydrated condition. In the beginning of this study, many animals had died in vain because of too late start of i. v. fluid. But it can be stressed that all rabbit without exception developed cholera like diarrhea. We have already used this model for many experiments without failure to develop diarrhea.

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成熟家兎における実験コレラの新型モデル

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成熟家兎における実験コレラの新型モデルを開発した．径約6 mm のシリコンチューブを，回盲部から小腸へ向って約10cm挿入し，回腸末端を結紮する．コレラ菌約10^6個を十二指腸へ注入すると平均12時間で下痢便がチューブを通して吸引されるようになる．下痢が始まったら直ちに5％ブドウ糖入りの乳酸化リンゲル液で持続点滴を始め（耳静脈から），下痢量＋100～200ml の割合で輸液を続ける．この方法で，体重2～3 kg のウサギから1時間に20～30ml，1日500ml 以上の下痢便を採取することが出来る．発症率（再現性）は100％であった．実験終了後，チューブを除去してウサギを生かし続けることも可能である．このモデルにおいて経時的に採取される便は盲腸・結腸において修飾されていない．従ってその分析（菌叢・抗体・酵素・細胞成分・胆汁成分・電解質・等々）によってコレラの小腸で何が生じているかを判定することが出来る．また人工的に投与した物質の追跡によって吸収・分泌能などを判定することも出来る．更に脱水状態からその回復期における生理的反応，免疫の成立経過等々，このモデルの利用価値は極めて高いものであり，我々も既に抗生剤の腸内移行，コレラトキシンの作用時間などを検討するために使用している．

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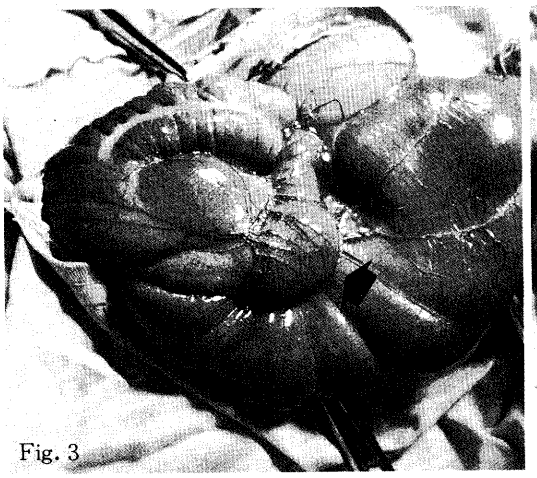
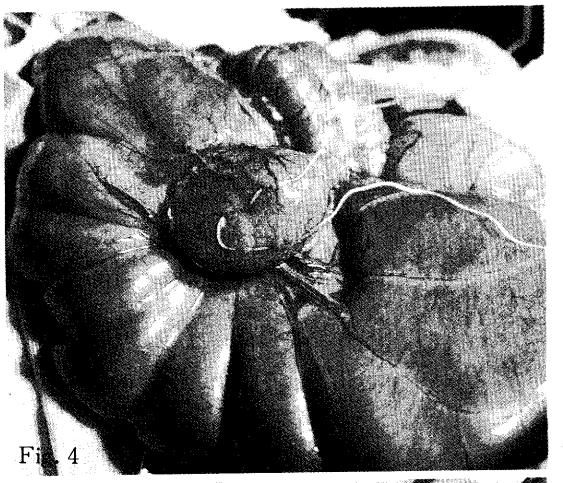


Fig. 3



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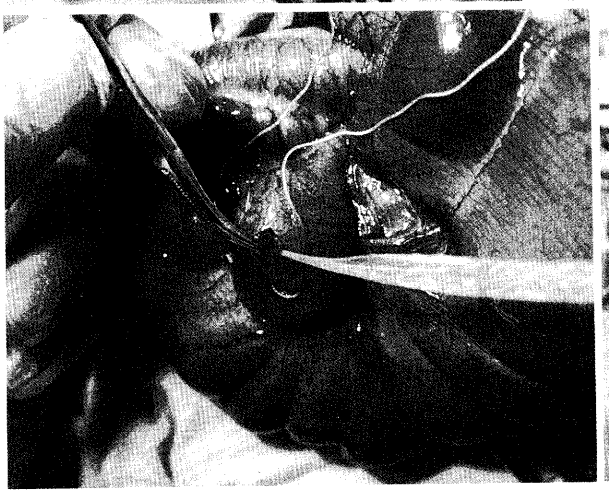


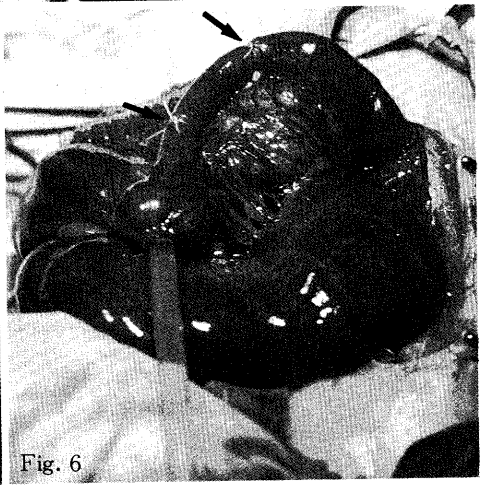
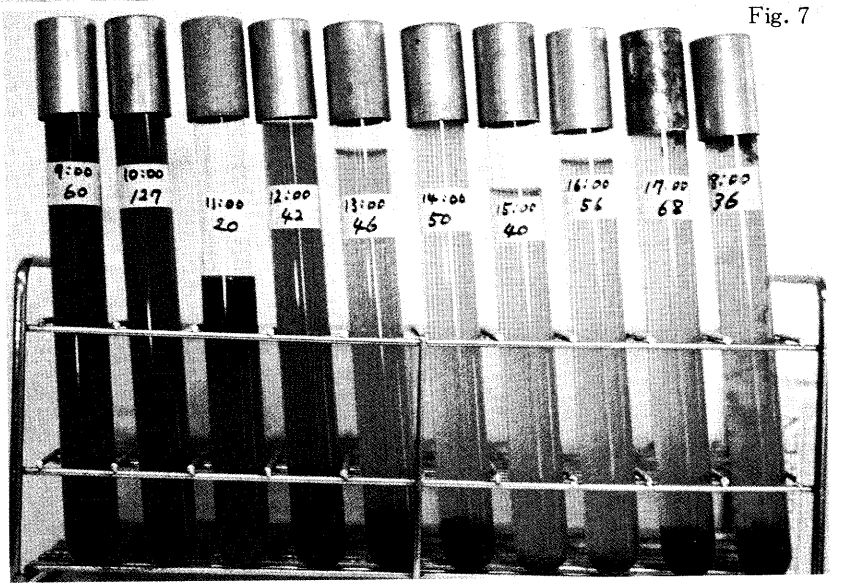


Fig. 6

Fig. 7



Figs. 3-6. Actual procedure to put the tube in position.
3. The appearance of ileo-caecal junction (arrow).
4. A silk was put circularly through subserosa at the ileo-caecal junction, prior to make an incision. 5. A small incision was made in the circle of the silk, and the tube was inserted into the ileum. 6. The tube went up to the ileum, and the tip was located at about 10 cm from the incision. The terminal ileum with the tube was tied with silk (arrows) to fix the tube and to block the movement of the intestinal contents between the ileum and the cecum.

Fig. 7. The appearance of sucked diarrheal fluid. The time of suction and the volume (ml) were indicated in each test tube. In the beginning, the stool contained much amount of food sediments.